ACTION OF SALTS ON *FUNDULUS* EGG.

I. The Action of Na, K, and Ca Chlorides upon the Egg of *Fundulus*.

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Interest in the fundamental action of chemicals upon the egg, egg membrane, embryo, and larva of the marine fish *Fundulus* has centered largely around the work of Loeb and his associates (1–17). The results of this work seem to show that marked differences exist in reactions of the eggs and hatched embryos or larvæ to different salts. Marked degrees of salt antagonism are also pointed out. Inasmuch as the conclusions drawn from this work are based almost entirely upon the egg and hatched embryo or larva, it was thought highly desirable to recheck the observations, using eggs and embryos dissected out of the eggs, as well as hatched embryos or larvæ. By means of an operative technic devised by Doctor J. Nicholas [1] of Yale University, the details of which will be published elsewhere, it has been found possible to remove the outer egg membrane from the embryo and yolk sac without, in any apparent way, interfering with the further development of the embryo. As a matter of fact, embryos removed from the egg develop in sea water in as normal a way as those enclosed in the egg. It has thus been possible to compare the action of various salts directly upon the egg, the embryo dissected free from the egg membrane, and the larva hatched from the egg.

That Loeb conceived of a similarity in the action of salts upon the hatched embryo or larva and the embryo contained in the egg can easily be gathered from the following passages. In 1911 he says (6): ". . . if the fish is *out of the shell* the addition

[1] The writer is deeply indebted for the assistance rendered by Doctor J. Nicholas who was kind enough to carry out the operative technic involved in removing the egg membrane.

of $CaCl_2$ alone is no longer sufficient and the addition of KCl also becomes necessary." Other remarks in the same article seem also to indicate that the marked differences in the reactions to salts between the larva and embryo were not appreciated. In 1916 he says (8): "This prolongation of life through the addition of Ca is due not to an action upon the protoplasm but to a prevention of the diffusion of the NaCl into the egg, since if we take the embryo out of the egg (or use the newly hatched embryo) it is killed in 50 cc. 3 *M* NaCl + 1 cc. 10/8 *M* $CaCl_2$ inside of a few minutes." In his last paper on *Fundulus* (17) he says: "In 1905 the writer suggested as an explanation that a pure NaCl solution, if its concentration exceeded a certain limit, made the membrane of the egg more permeable, so that NaCl could diffuse into the egg, killing the embryo, while this increase in permeability was prevented by the presence of a low concentration of Ca." These and similar passages throughout his works seem to the writer to show that the marked differences in the reactions to salts between the embryo dissected out or freed from the egg membrane and the larva were not fully taken into consideration.

The present paper embodies results obtained from investigations carried out during the summer of 1927 at the Biological Laboratory, Cold Spring Harbor, on the effects of Na, K, and Ca chlorides on the eggs, embryos freed from the egg membrane, and larvæ of *Fundulus heteroclitus*.

Methods.

The eggs were stripped directly from the female fish into finger bowls containing sea water and then fertilized. They were kept at room temperature (20°–25° C.) as well as at lower temperatures (15°–18° C.). Those at the lower temperatures naturally took longer to reach a given stage in development than those kept continuously at the higher temperatures. The eggs were usually washed for varying periods in distilled water before use in order to free them of adhering salts as suggested by Loeb (13–17). This procedure, however, seems to the writer necessary to but a limited degree, since similar reactions are given by eggs washed for varying periods except when the time factor is

greatly lengthened, *i.e.*, to several days as was the case in many of Loeb's experiments (13–17).

The egg membrane was cut mid-laterally by means of fine pointed iridectomy scissors and the contained embryo gently rolled out of the shell by means of a fine probe. Eggs in as nearly as possible the same stages of development were used together with embryos dissected from similar eggs. It is of considerable importance that eggs and embryos be in the same stages of development, since marked age differences in reactions to some salts seem to exist. The length of exposure of the egg to water is also an important factor in changing the general consistency of the egg membrane. Both eggs and embryos were placed in covered Syracuse watch-glasses in 10 cc. of the solution and were constantly observed under a compound microscope during the course of the experiments. Five to ten organisms were used in a single watch-glass. It is of much importance to carry through experiments until the eggs hatch or the organisms die since in many cases the eggs live in certain solutions but upon further development and hatching the embryos quickly succumb. The embryos and eggs used ranged in developmental stages from those in which heart action was just beginning to those with fully developed circulation and ready to hatch. Newly hatched larvæ were used for comparison with the dissected-out embryos and eggs. The end-point observed and recorded in all the experiments herein reported was the time of cessation of the heart beat. Recovery of the heart beat in sea water was also noted but will be dealt with in a subsequent communication. The salts used in these experiments were c.p. NaCl, $CaCl_2$, and KCl made up in distilled water. Only the effects of normal solutions of these salts will be presented at this time since the results at this concentration are typical.

Results of Experiments.

Since in general the results of all experiments are qualitatively alike only typical experiments will be described.

N KCl.—KCl, as repeatedly pointed out by Loeb and his coworkers (1–17), acts with considerable rapidity on the egg, dissected-out embryo, and larva. In all, the heart quickly

TABLE I.

Salt.	Egg.	Embryo Freed from Membrane.	Larva.	Remarks.
N KCl.....	+ (75 min.)	++ (15 min.)	+++ (4–6 min.)	Penetrates membrane rather slowly
N $CaCl_2$.....	+ (98 min.)	+ (51 min.)	++ (30 min.)	Penetrates membrane rather slowly
N NaCl.....	young + (24 hrs.) older ○	○	+++ (10 min.)	Penetrates membrane rather slowly
Equal parts:				
N NaCl + *N* $CaCl_2$..	○	+ (12–24 hrs.)	++ (22 min.)	No penetration
N KCl + *N* $CaCl_2$...	++ (30 min.)	++ (20–25 min.)	++ (20 sec.)	Aids penetration
N NaCl + *N* KCl...	+ (73 min.)	++ (38 min.)	+++ (15 min.)	Penetrates membrane

Explanation.—Shows general conception as to toxic action of salts upon eggs, embryos and larvæ. + = relatively slow toxic action; + + = a faster toxic action; +++ = a marked toxic action; ○ = non-toxic. Figures show average time for cessation of heart beat.

stops beating, the relative order of resistance being, larva < embryo < egg. The egg is about 4 times as resistant as the dissected-out embryo, and about 20 times as resistant as the larva. In the action of KCl upon the embryo within the egg, about three fourths of the time necessary to cause cessation of the heart beat is spent in the passing through the egg membrane (Table I.). Eggs with embryos in which the heart is just beginning to beat appear less resistant then older eggs.

N $CaCl_2$.—$CaCl_2$ is also extremely toxic for eggs, embryos, and larvæ, the relative order of resistance being larva < embryo < egg. The egg is about one half as resistant as the embryo, and about 3 times as resistant as the larva. Relatively less time is spent by the Ca in going through the egg membrane than by K, since the embryo outside of the egg is killed in less than one half of the total time required for cessation of the heart beat of the embryo within the egg membrane. No marked age differences in resistance to $CaCl_2$ seem to exist, since the organisms are killed at all embryonic stages in almost the same relative time.

N NaCl.—The action of NaCl is by far the most interesting of those studied, since the results obtained are quite different from those reported by Loeb (17). The relative resistance to NaCl is, larva < embryo < egg. This series, however, must be modified because with NaCl the question of age enters in a most amazing way. Freshly fertilized eggs, as pointed out by Loeb (2), are killed in solutions of NaCl. As the egg grows older its resistance to NaCl increases up to the time of hatching. Eggs in which the embryonic heart has just begun to beat are susceptible to NaCl, while the same embryo removed from the egg will usually live for days in the same solution. As a matter of fact, they live almost as long as it takes for the normal embryo in sea water to reach the time of hatching. The freshly hatched larvæ, however, are killed very quckly when put into the same NaCl solution (Table I.). A marked change in the resistance of the animal to NaCl thus takes place when the embryo is ready to emerge or emerges from the egg.

Equal parts N KCl + N $CaCl_2$.—In such a mixture the toxicity for the egg, embryo, and larva is about the same (Table I.).

The time taken to cause cessation of the heart beat, in the mixture however, is quite different from that required in the case of solutions of the individual salts, as is shown in Table I.

Equal parts N NaCl + N $CaCl_2$.—In this mixture the larva is killed in approximately 20 to 25 minutes. The embryo dissected from the egg membrane can survive from 6 to 24 hours while the eggs are not killed. As a matter of fact, eggs will hatch in the solution, and the hatched larva is quickly killed, showing quite conclusively that while in the egg the embryo is protected; once out, it quickly succumbs. The embryo dissected from the egg membrane is always found to be much more resistant than the larva (Table I.).

Equal parts N NaCl + N KCl.—Such a mixture is quite toxic for the egg, embryo, and larva, the relative resistance being: larva < embryo < egg. The egg is approximately 2 times as resistant as the embryo and about 5 times as resistant as the larva (Table I.).

Discussion.

The above results seem to the author to be of interest inasmuch as they definitely point out and show that any assumption as to the interior condition of the egg cannot be relied upon until satisfactorily tested and proven. Loeb (1–17), in most of his work on *Fundulus*, seems to have assumed that the resistance of the hatched embryo or larva was comparable to that of the embryo within the egg membrane. In the case of NaCl in particular this view is shown to be erroneous, and it is quite possible that further investigation will yield equally interesting results. That the egg membrane changes in consistency with age is quite apparent in the ease with which the membrane can be cut. Young fertilized eggs have membranes quite tough and turgid; eggs exposed to distilled water and at low temperatures for long periods of time have much softer and more pliable membranes. Associated with these structural changes are doubtless the marked physiological ones observed. Further proof of marked physiological changes in the egg membrane have come out of unpublished investigations recently conducted by Miss E. Yagle of this Laboratory, on the exosmosis of H_2O from the *Fundulus* egg of different ages. Loeb (6), also points out in

several instances that such changes occur—*e.g.*, ". . . since the *newly fertilized* egg is killed more rapidly by a *m*/2 solution of NaCl than it is killed by the same solution one or two days after fertilization." Young eggs, therefore, are very susceptible to NaCl while older ones are quite resistant. Young embryos, on the other hand, are much less susceptible than are very old ones. The hatched embryo or larva, however, is extremely susceptible to NaCl. The question as to the fundamental action of NaCl upon the younger eggs with hearts just beginning to beat apparently is not one of a purely chemical nature but rather of an action on the membrane in which the embryo is doubtless killed by some secondary effects, possibly osmotic. In eggs thus killed the contained embryo always appears much shrunken in size.

By means of the dissection technic in liberating the embryos from the egg it is possible to study quantitatively the relative effects of the salt upon the membrane and contained embryo and also upon the embryo free from the egg membrane. KCl, for example, seems to spend about 75 per cent. of its total time effect upon the egg membrane and approximately 15–50 per cent. on the embryo. $CaCl_2$ on the other hand, spends about 60 per cent. on the membrane and 30–40 per cent. on the embryo. NaCl must exert most of its effect on the membrane in those cases in which it is toxic since for embryos removed from the egg it is relatively non-toxic.

The site of action of the three salts Na, K, and Ca chlorides has always been of considerable physiological interest. Loeb (17), in the case of *Fundulus*, seems to have attributed the fundamental action of these salts to the membrane—the process being purely a diffusion phenomenon. In young eggs NaCl perhaps does not penetrate the membrane while in older eggs the membrane seems freely permeable, since the contained embryo is not killed in the same solutions when removed from the egg. KCl and CaCl seem to penetrate the egg membrane, the Ca entering in a relatively slightly shorter time than the K. The Na + Ca antagonism as suggested by Loeb (2) must be a membrane phenomenon since the embryo removed from the egg or the hatched larva is quickly killed in such a mixture. Combi-

nations of Na + K, however, act more like KCl alone and little if any antagonism seems to exist (Loeb, 1, 2). The combination K + Ca, on the other hand, seems to exert additive effects and to kill eggs and embryos in almost the same time.

Several additional facts noted in these experiments are of especial interest since they seem intimately concerned in any fundamental explanation of the salt effect upon the egg and embryo. The space between the egg membrane and embryo is at first very small, due to the large size of the yolk sac. As the embryo develops rather a large amount of fluid accumulates between the egg membrane and embryo. This increase in fluid perhaps has much to do in modifying the rates at which the embryo is killed while in the egg. Around the yolk sac and embryo there is also a delicate vitelline membrane, the properties of which seem of much importance in respect to the resistance of the embryo to various salts. If with a very fine pointed needle a minute hole is made in this membrane immediately ventral to the eye and the embryo, still normal, transferred to a solution of NaCl or left in a solution of NaCl which is not ordinarily toxic, it is quickly killed. It seems that this membrane is an important factor in determining the resistance of the embryo to NaCl. Further investigations are to be carried out on these points.

Summary.

(1) By means of an operative technic it is possible to remove the egg membrane from the egg of *Fundulus heteroclitus* and to compare experimentally the action of salts on the egg, the embryo freed from the membrane and the newly hatched larva.

(2) The effects of normal solutions of K, Na, and Ca chlorides upon the above are reported.

(3) The embryo, freed from the egg membrane, is quite resistant to NaCl solutions while the hatched larva is quickly killed in the same solution.

(4) The resistance of the eggs to NaCl increases with age.

(5) K and Ca chlorides kill the dissected-out embryo much more quickly than the egg, while the recently hatched larva is much more sensitive to the two salts than is the embryo.

(6) Combinations of these salts show antagonistic action.

Na + Ca mixtures are not toxic for eggs but are markedly so for the embryo freed from the egg membrane and for the newly hatched larva.

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